ORIGINAL RESEARCH

The effectiveness of deoxyshikonin as a treatment for oral squamous cell carcinoma via inhibition of the AKT1/mTOR signaling pathway: A combined bioinformatic analysis and experimental validation

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Abstract

Context: Oral cancers, particularly oral squamous cell carcinoma (OSCC), are aggressive malignancies with early lymph node metastases. Deoxyshikonin (DSK), a naphthoquinone derived from Arnebia euchroma, exhibits anticancer properties with minimal toxicity.

Aim: The primary objective is to evaluate DSK's therapeutic potential in OSCC by targeting the AKT1/mTOR signaling pathway, which regulates cancer cell proliferation and survival.

Materials and Methods: Growth inhibition of KB cancer cells was evaluated using the MTT assay. DSK's effects on oxidative stress were measured by SOD, CAT activity, and ROS detection via DCF-DA staining. Morphological changes in KB cells were analyzed using PI, DCF-DA, and AO/EtBr staining. RT-PCR assessed mRNA expression changes in key apoptotic and mTOR pathway genes. Key gene targets (SIRT1, MCL1, CASP3, CASP9, IL6, IL10) were identified using CTD and Gene Cards. Network analysis revealed enrichment in FoxO, p53, and inflammatory mediator signaling pathways. Molecular docking studies assessed DSK's binding affinity to target proteins (SIRT1, CASP9, IL6, IL10).

Results: DSK treatment upregulated apoptotic markers CASP3 and CASP9 while downregulating mTOR pathway targets IL6, IL10, SIRT1, and MCL1. Docking studies showed strong binding affinity between DSK and key proteins. DSK also induced oxidative stress and disrupted metabolic homeostasis. MD simulations showed strong stability for protein target against DSK.

Conclusion: DSK effectively suppresses AKT1/mTOR signaling in OSCC, demonstrating its potential as a therapeutic agent. This study provides a foundation for future preclinical and clinical research.

Keywords: Oral squamous cell cancer, deoxyshikonin, biological targets, therapeutic pathways, experimental confirmation, and bioinformatics analysis.

Introduction

As the sixth most frequent cancer worldwide, head and neck carcinoma primarily have oral squamous cell carcinoma (OSCC) as its histological type. Treatment options for patients with head and neck squamous cell carcinoma (HNSCC) mostly consist of surgery, radiation therapy, and chemotherapy[1]. In developing nations, approximately 95% of oral malignant lesions are caused by OSCC[2]. In Taiwan, drinking, smoking, and eating betel nuts are the most prevalent risk factors for oral cancer[3]. Aggressive tumors with varied degrees of differentiation and early-stage widespread lymph node metastases are its defining characteristics[4,5].

Shikonin is a naturally occurring naphthoquinone with anti-inflammatory and anti-tumor properties. Because of its toxicity in the body, oral shikonin is rarely given directly in therapeutic settings[6]. Deoxyshikonin (DSK) has been demonstrated to use several interconnected mechanisms to exhibit a wide range of oncostatic effects in a variety of malignancies. Research shows that DSK suppresses the growth of carcinoma of the colon by decreasing the activity of the PI3K-Akt-mTOR system[7]. DSK has been demonstrated to suppress glycolysis and cancer cell viability in acute myeloid leukemia, along with decreased pyruvate kinase M2 expression and activity[8]. The high level of interaction with related proteins was

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discovered by the CPEB3 gene and protein interaction. The expression of CPEB3 in HNSCC results in the down regulation of cell population growth, the negative regulation of cytoplasmic translation, and the cellular response to amino acid stimulation. Notably low expression of CPEB3 affects patient survival with HNSCC ($p = 0.013$; $p<0.05$). Compared to healthy controls, HNSCC patients with altered mTOR pathway expression had decreased levels of CPEB3. NEURL1 and IPO5 genes shows high interaction with CPEB3 using STITCH database. It is imperative that more studies be done since CPEB3 is essential to the development and growth of HNSCC tumors. Thus, it is postulated that CPEB3 may contribute to the carcinogenesis of HNSCC by inhibiting the expression of the CPEB3 gene and affecting the mTOR pathway[9]. The aim of this work is to assess the antitumor effects of deoxyshikonin on oral squamous carcinoma cells (KB cells). More extensive investigation and clinical trials are needed to completely evaluate the therapeutic potential of this medication in treating oral squamous cell carcinoma. It is crucial to ascertain the efficacy and safety of DSK since this could lead to opportunities for its incorporation into oral cancer treatment regimens.

Materials and Methods

Identification of potent DEGs interacting with deoxyshikonin

A comprehensive analysis of the dataset was undertaken utilizing the tools comparative toxicogenomics database (CTD), and Gene cards. OSCC-related targets and deoxyshikonin were analyzed using platforms, and Fun Rich V3.1.3 software was utilized to visually show the results[10]. A Venn diagram was produced by the Fun Rich tool, providing insights into the intricate biochemical landscape of cancers of the mouth.

Pharmacological network construction of deoxyshikonin from STRING database

DEGs from OSCC datasets and the STITCH database were analyzed using Cytoscape to construct DPI and PPI networks, identifying key molecular pathways in oral cancer[11,12].

Gene Ontology and enriched pathway analysis for deoxyshikonin

Functional and an analysis of pathway enrichment using g and KEGG revealed the biological functions and enriched networks of up-regulated DEGs, offering insights into the physiological effects of these specific genes in tumors of the mouth.

Molecular docking

Using structures from the Protein Data Bank, the study investigated deoxyshikonin's binding relationships with proteins MCL1, SIRT1, IL6, IL10, CASP3, and CASP9. The Lamarckian genetic algorithm (LGA) was employed to perform binding simulations using PyRx software. Discovery Studio 2021 was used to evaluate and illustrate the docking data in both 2D and 3D 13.

Cell lines

Under CO_2 incubator conditions, these KB cell lines were grown at 37°C in a regulated, humid atmosphere. A 10% fetal bovine serum (FBS) supplement and 1% penicillin-streptomycin antibiotic were added to Dulbecco's Modified Eagle's Medium (DMEM) to promote cell proliferation.

Cell viability assays

The KB cell line was used to test the cytotoxicity of deoxyshikonin utilizing the MTT and trypan blue exclusion tests[14]. MTT reagent was added after an initial population of $1x10^4$ cells was cultured for one night. In order to determine the vitality of the cells, the resultant formazan was solubilized with DMSO and its absorbance was measured at 590 nm. For the trypan blue assay, following exposure to deoxyshikonin, the cells were collected through trypsinization. These collected cells were mixed with a 0.4% trypan blue solution in a 1:1 ratio. After a brief incubation of 5 minutes at room temperature, cell counting was performed using a hemocytometer chamber.

Metabolic assays

Following a 24-hour treatment with deoxyshikonin, KB cells were washed with KRPH buffer, and glucose absorption was assessed using a colorimetric kit. Using colorimetric assays, lactate levels and enzyme activity were measured.

Measurement of cellular SOD activity

Using a SOD Assay Kit, the amount of Superoxide Dismutase (SOD) in KB cells treated with deoxyshikonin for 48 hours was assessed. Measurements of absorbance were made at 450 nm following the production of a water-soluble formazan dye.

Measurement of cellular catalase activity

The activity of the cellular catalase enzyme was assessed using a catalase assay kit obtained from Sigma-Aldrich. In brief, KB cells at a concentration of 1x106 were treated with deoxyshikonin for 24 hours. After treatment, the cells were washed with PBS and lysed, and the resulting lysates were collected. These samples were then mixed with an appropriate volume of assay buffer (50 mM KH_2PO_4/50 mM Na_2HPO_4, pH 7.0) in a microcentrifuge tube. A 200 mM H_2O_2 solution was added to start the reaction, which was then incubated for five minutes at 25°C before a stop solution was added. The combination was then incubated for a further ten minutes at 25°C.

Measurement of intracellular ROS activity by DCFH-DA

The amount of intracellular ROS produced was measured using the fluorescent dye probe DCFH-DA. In short, $1x10^4$ KB cells were pre-treated with deoxyshikonin in a time-dependent manner after being seeded one day earlier. Following this, cells were treated with PBS and incubated with 10 μM DCFH-DA for 15 minutes at 37°C in the absence of light.

Immunofluorescence staining by PI and DCFH-DA staining

The morphological changes of KB cells by treating deoxyshikonin was visualized by immunofluorescence staining. A 5-μM concentration of the non-fluorescent intracellular probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) was utilized to evaluate the formation of ROS within cells. In the experimental setup, $5x10^5$ of KB cells were treated with deoxyshikonin for 40 minutes. Subsequently, DCFH-DA was introduced, and the cells were cultured in the absence of light for an additional 20 minutes at 37°C. Following this incubation period, cells were resuspended in 1 ml of ice-cold phosphate-buffered saline (PBS). The oxidative activity of ROS on DCFH was visualized

using Olympus fluorescence microscopy (Tokyo, Japan) with a Zenoptik camera.

Nuclear staining by AO/EtBr

The aim of this study was to evaluate and identify apoptotic cells through nuclear staining using acridine orange (AO) and ethidium bromide (EtBr), based on characteristic changes in nuclear morphology and membrane integrity. During the experiment, 10µl of AO/EtBr solution was added to the KB cells that had been treated with deoxyshikonin and spread equally by covering the sample with a coverslip. Using an upright fluorescent microscope, apoptotic cells could be distinguished by their distinctive red fluorescence, which was caused by condensed chromatin and broken nuclei. Conversely, normal cells exhibited green fluorescence, and their presence was observed (20× magnification) using Olympus fluorescence microscopy (Tokyo, Japan) and quantified in Image J software.

Gene expression by RT-PCR

An average density of 5 × 10^6 cells per well was achieved by plating KB cells in a 6-well dish. They were then cultured for an additional night before being extracted from their RNA using Abgene's TRIR. Spectrophotometric measurement yielded the RNA content in micrograms (µg)[15,16]. Using Takara SyBr green master mix and specially created primers targeting IL6, IL10, CASP3, CASP9, MCL1, and SIRT1, a reaction combination was created for the Real-Time PCR measurement of mRNA expression.

Molecular Dynamics simulations

Protein-ligand interactions were simulated using GROMACS with molecular dynamics (MD). The protein-ligand complex, dissociated in PyMOL, underwent topology construction in GROMACS using the Amber99sb-ildn force field for the protein and ACPYPE for the ligand. The combined topology was placed in a dodecahedron box (1.0 nm) solvated with TIP3P water, and neutralized with NaCl. Energy minimization (1000 steps) was followed by equilibration at 300K (NVT, 300 ps) and 1 bar (NPT, 500 ps) using parameters from the Lekumal tutorial. A 10 ns MD simulation was performed, and the trajectory file (XTC) was saved for analysis[17].

Statistical analysis

The data was analyzed using R Script for bioinformatic analyses. mRNA expression analysis was done by ONE-WAY-ANOVA using GraphPad Prism 8's software followed by log-rank test and Kaplan-Meier cure were calculated accordingly HR and p value. Significance was considered at the levels of $p<0.05$.

Results

Pharmacological networking strategy of deoxyshikonin

A Venn diagram revealed 38 genes that were consistently present in OSCC datasets, indicating that they might be related to deoxyshikonin. MCL1, SIRT1, IL6, IL10, CASP3, and CASP9 were the six main targets identified by network analysis utilizing the STITCH database. By highlighting these targets' importance in OSCC, this work reinforces the link between deoxyshikonin and them (Figure 1A-D).

Functional annotations associated with deoxyshikonin in OSCC

A bubble chart was used to illustrate the data and emphasize the most important functional insights (Fig. 2A). It has been found that these targets regulate essential biological processes such as apoptosis, gene expression regulation, cell division, and the glycolytic process. These targets have

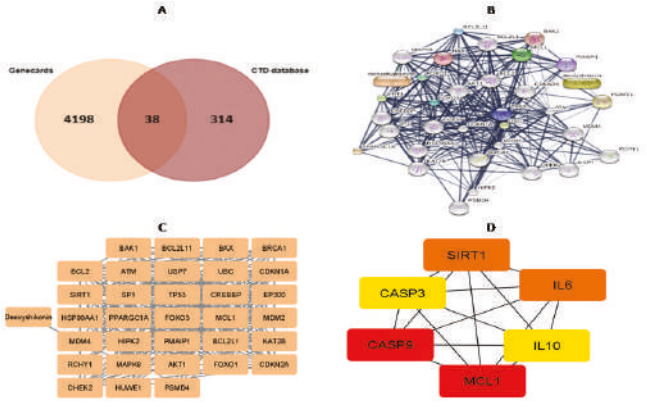

Fig. 1: Pharmacological network analysis of deoxyshikonin is connected to potent OSCC targets. (A) A Venn diagram produced using the Fun rich tool shows how the OSCC regulating targets from Genecards and the frequently overlapping key targets for deoxyshikonin regulation from the CTD database overlap. (B) STITCH, an online drug-protein interaction networking tool, was used to demonstrate the material's high association with its key targets. (C) The relationships between proteins are shown using the Cytoscape tool. (D) The Cytohubba plugin for Cytoscape additionally displays the most important six significant goals.

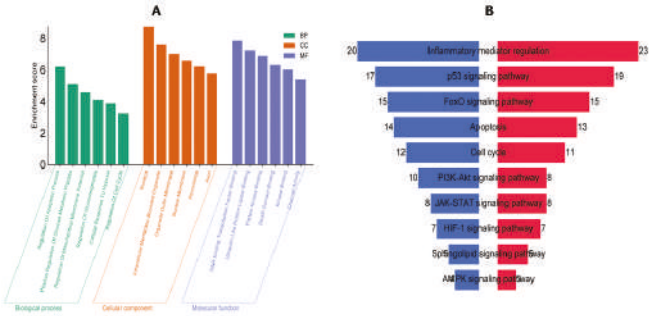

Fig. 2: The OSCC dataset's enriched route and gene ontologies for significant targets are displayed. The gene ontology for physiological processes, biological elements, and biochemical functions were displayed in a bubble chart (A and B). Special emphasis was placed on metabolic processes, environmental information processing, cellular processes, organismal systems, and human disorders. Furthermore, a high degree of enrichment for these pathways was shown by the KEGG pathway analysis.

been associated with the intracellular membrane, boudered organelle, and nuclear membrane. Protein kinase binding, ubiquitin-like peptide kinase binding, and DNA-binding gene transcription factor interaction were a few of their biological functions. KEGG pathway analysis provided additional information, demonstrating a high enrichment in pathways like FoxO signaling, PI3K-Akt pathways, p53 signaling, and inflammatory mediator control. Figure 2B Our knowledge of deoxyshikonin-associated targets and the intricate web of dysregulated pathways in oral cancer has improved as a result of the analysis, which showed that numerous target genes were enriched in pathways connected to cancer.

Deoxyshikonin inhibits cell growth and aerobic glycolysis in KB cells

Finding safe and effective therapeutic medications is made more likely by the combined synergies between computer analysis and experimental research, including investigations of cell lines. This accelerates the drug-development process. We thoroughly assessed the cytotoxic effects of deoxyshikonin on KB cells in the setting of oral cancer using the MTT and trypan blue tests. DSK was given to KB cells over a period of 24 and 48 hours at various doses (20–100 μM). Deoxyshikonin administration over time in KB cells led to a significant and decrease in viability, as indicated by the results, which are displayed in Fig. 3A. Furthermore, deoxyshikonin administration for 24 and 48 hours decreased the growth of KB cells, as seen by the trypan blue assay (Fig. 3B), which further supported the results. Further evidence that lactose production and glucose absorption in KB cells are blocked comes from the time-dependent injection of DSK in Figures 4A and B. Overall, these results show that DSK inhibits aerobic glycolysis and kills oral cancer cells.

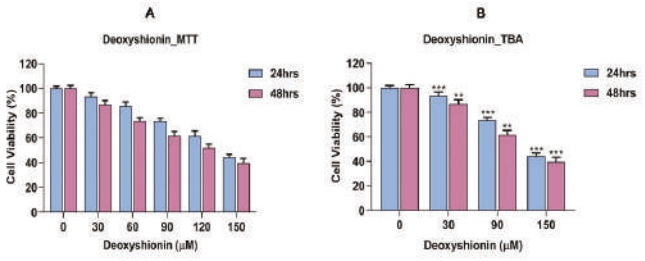

Fig. 3: The proliferation of KB cells is inhibited by deoxyshikonin (A and B). The increasing deoxyshikonin concentration (0–100μM) over 24-48 hours intervals is demonstrated by cell viability as assessed by MTT and trypan blue tests. The data were analyzed by ONE-WAY-ANOVA using GraphPad Prism 8's software. Significance was considered at the levels of p<0.05. **- significance at the levels of p<0.01; ***- significance at the levels of p<0.001; ****- significance at the levels of p<0.0001.

Deoxyshikonin possess anti-oxidant activity in KB cells

Superoxide dismutase (SOD) and catalase (CAT) activity in cells were also measured; as seen in Fig. 5(A and B), both activities revealed lower levels suggestive of deoxyshikonin's impact on ROS activity in oral cancer cells. In addition to expanding our knowledge of deoxyshikonin's impact on ROS activity, this experimental approach emphasizes the compound's possible significance in the treatment of oral cancer. DCF-DA staining (Fig. 5C) demonstrated increased ROS activity following deoxyshikonin injection, highlighting the importance of ROS regulation in the growth of cancer cells. The assessment of CAT and SOD activity provides additional evidence for the role deoxyshikonin plays in controlling ROS in KB cells.

Morphological changes of KB cells by treating deoxyshikonin

We administered KB cells with the IC50 concentrations of deoxyshikonin for 24 and 48 hours, monitoring any changes in cytomorphology, to verify the drug's efficacy. The results demonstrated that the size and characteristics of the untreated cells remained unchanged. In contrast, significant cytomorphological changes, including cell shrinkage, blebbing, and instances of cell death, were visible when the treated cells were stained with PI and DCFH-DA. The outcomes demonstrated that when DCFH-DA dye is used, untreated KB cells exhibit decreased ROS activity. Furthermore, nuclear

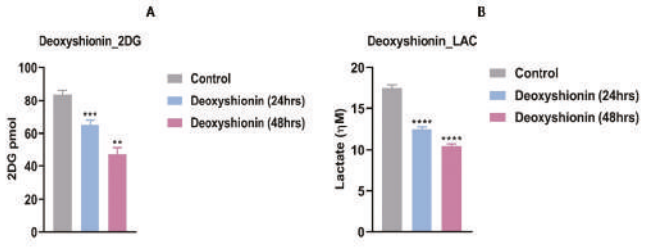

Fig.4: Deoxyshikonin stimulates aerobic glycolysis in KB cells. A and B-The amount of lactose generated and glucose absorbed by KB cells was measured 48 hours after they were treated with deoxyshikonin. The data were analyzed by ONE-WAY-ANOVA using GraphPad Prism 8's software. Significance was considered at the levels of p<0.05. **- significance at the levels of p<0.01; ***- significance at the levels of p<0.001; ****- significance at the levels of p<0.0001.

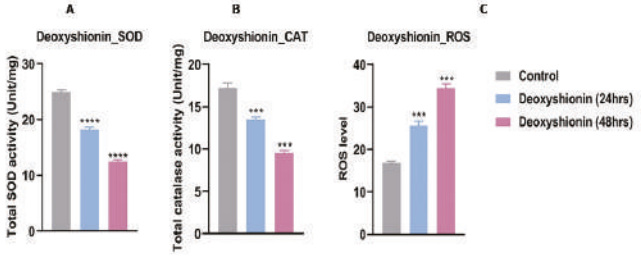

Fig. 5: Deoxyshikonin exhibits antioxidant qualities in KB cells. (A-C)- Cellular SOD, CAT, and intracellular ROS were assessed by DCF-DA after treating KB cells with deoxyshikonin. significance level. The data were analyzed by ONE-WAY-ANOVA using GraphPad Prism 8's software. Significance was considered at the levels of p<0.05. **- significance at the levels of p<0.01; ***- significance at the levels of p<0.001; ****- significance at the levels of p<0.0001.

staining with AO/EtBr was used to ascertain the apoptotic rate of KB cells. Notably, DSK treatment of KB cells over a 24- and 48-hour period caused KB cells to undergo both early and late apoptosis, as Figure 6 illustrates. Notably, the group treated with deoxyelephantopin at 48 hours showed a significant number of cells passing through both early and late apoptotic processes. The potential therapeutic efficacy of deoxyshikonin in the treatment of oral cancer is increased by these data, which offer significant insights into the mechanisms of oxidative stress, apoptosis, and cellular responses connected to the treatment.

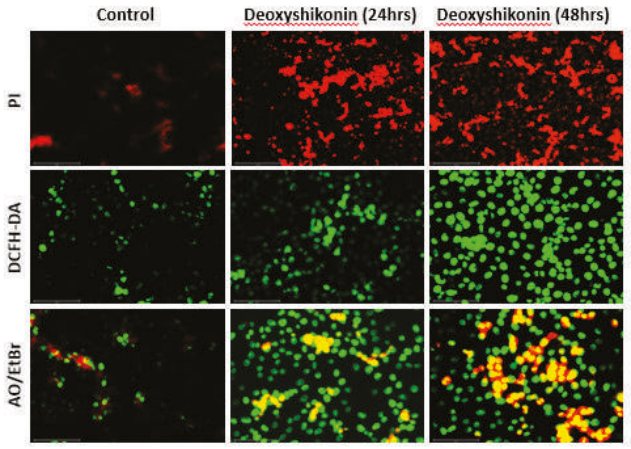

Fig. 6: The impact of deoxyshikonin on KB cells' ROS activity and apoptosis. After applying deoxyshikonin to KB cells for 24 and 48 hours, the morphological changes were detected using immunofluorescence labeling. at 20X magnification, DCFH-DA for intracellular ROS activity, PI for nuclei staining, and AO/EtBr labeling for early and late apoptosis in KB cells. The photos were quantified using ImageJ and shown by Olympus using a Zenoptik camera.

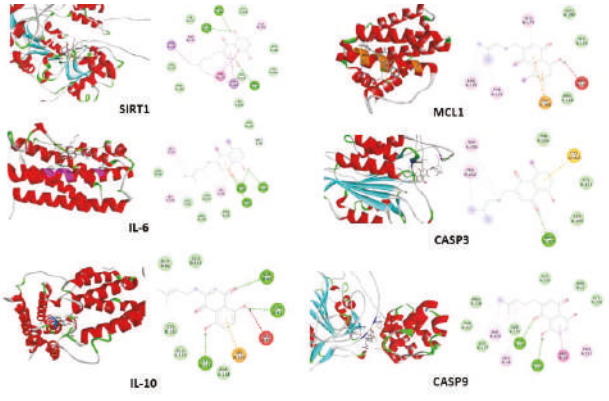

Fig.7: Molecular docking links deoxyshikonin with significant OSCC targets, as illustrated. PyRx software is used to do a molecular docking analysis to show deoxyshikonin's binding affinity for MCL1, SIRT1, IL6, IL10, CASP3, and CASP9 targets. The 3D and 3D structural representations discussed here were visualized with the help of the Biovia Discover Studio program.

Deoxyshikonin shows high binding association with AKT1/mTOR signaling targets

Results from molecular docking simulations shed light on the connections between deoxyelephantopin and significant proteins like MCL1, SIRT1, IL6, IL10, CASP3, and CASP9. Apoptosis, metabolism, and cell growth are all impacted by these proteins. As shown in Figure 7 and summarized in Table 1, the binding energies give valuable information about the strength of these interactions. When deoxyshikonin interacted with a number of significant proteins, including MCL1, IL6, SIRT1, CASP3, IL10, and CASP9, it exhibited notably high binding energies. Furthermore, a considerable number of binding interactions were observed between these targets and specific hydrogen-bonded amino acid residues, such as those linked to MCL1 (ARG187), IL6 (GLU200, PHE201, SER204), SIRT1 (GLN345, ASN346, ARG274), CASP3 (ARG207), CASP9 (TYR251, GLU321), and IL10 (CYS132, GLN81, SER84).

Deoxyshikonin facilitates AKT1/mTOR signaling targets in KB cell

Following the application of deoxyshikonin at IC50 values to KB cells for 24 and 48 hours, the study utilized real-time PCR techniques to evaluate the levels of messenger RNA expression for numerous enzymes critical for aerobic glycolysis and apoptosis. First, as can be observed in Fig. 8 (E and F), the administration of deoxyshikonin led to a notable increase in the mRNA expression of the apoptotic genes, regulating targets CASP3 and CASP9. This increase occurred within 24 to 48 hours. This group includes genes related to the inhibition of protein synthesis, cell division, aerobic glycolysis, and apoptosis, as shown in Fig. 8 (A, B, C, and D). These genes included IL6, IL10, SIRT1, and MCL1. These results suggest that DSK treatment in KB cells may impact the AKT1/mTOR signaling pathway and may have other effects.

Molecular stimulations

MD simulation analysis began with removing periodic boundary conditions (PBC) from the trajectory file (XTC).

Table 1: Deoxyshikonin molecular docking data with MCL1, SIRT1, IL6, IL10, CASP3, and CASP9

Compound	Protein(s)	Binding energy (Kcal/mol)	No. of amino acid residues	Amino acid residues
Deoxyshikonin (CID: 98914)	SIRT1	-9.1	3	GLN345, ASN346, ARG274
	CASP3	-6.3	1	ARG207
	CASP9	-7.1	2	TYR251, GLU321
	IL6	-7.0	3	GLU200, PHE201, SER204
	IL10	-6.9	3	CYS132, GLN81, SER84
	MCL1	-6.1	1	ARG187

Structural changes in DSK with protein ligand complexes were evaluated using RMSD (structural deviation from the starting point) and RMSF (residue fluctuations over time). RMSD graphs for protein complexes against DSK revealed high deviations due to loop regions. RMSF analysis showed significant fluctuations in the initial residues for protein. Overall, RMSD and RMSF results confirmed the stability of all protein ligand complexes during the MD simulations (Figure 9).

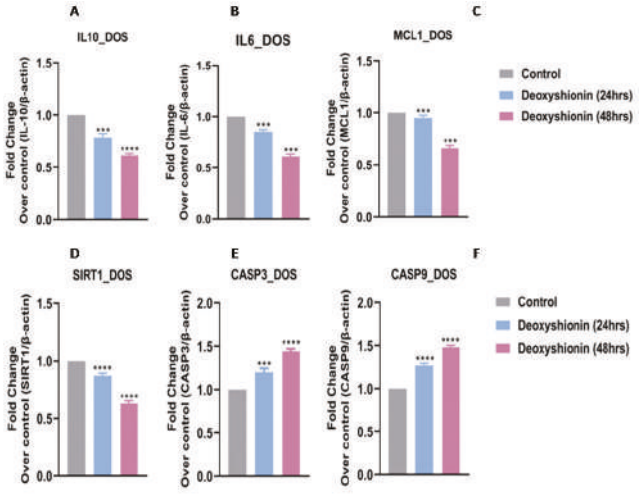

Fig. 8: In KB cells, deoxyshikonin suppresses the expression of key AKT1/mTOR signaling targets (A–F). q-RT-PCR was used to assess the effects of deoxyshikonin-potent main targets (MCL1, SIRT1, IL6, IL10, CASP3, and CASP9) on gene expression. The data were analyzed by ONE-WAY-ANOVA using GraphPad Prism 8's software. Significance was considered at the levels of p<0.05. **- significance at the levels of p<0.01; ***- significance at the levels of p<0.001; ****- significance at the levels of p<0.0001.

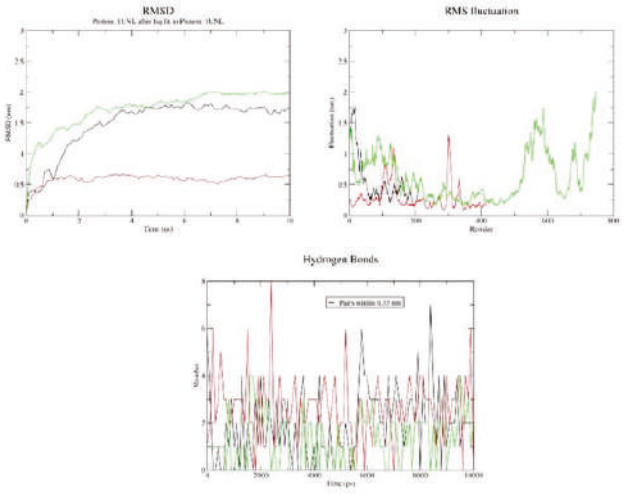

Fig. 9: Showing the molecular stimulation between protein ligand complexes.

Discussion

It has been demonstrated that one of the main causes of morbidity and death among tobacco and alcohol users is oral cancer, especially oral squamous cell carcinoma (OSCC) [18]. One of the most common types of oral cancer is called oral squamous cell carcinoma (OSCC), which has been linked to a number of carcinogens from oral behaviors like drinking alcohol, smoking cigarettes, and chewing betel quid [19]. Deoxyshikonin, with an IC_{50} value of 10.97 μM, exhibited significant anti-proliferative effects on HT29 cells. Upon treatment with deoxyshikonin at concentrations ranging from 0 to 50 μg/mL, the percentage of early apoptotic cells increased markedly, rising from 1% to 29%. The material also reduced the levels of the mTOR, Akt, p-Akt308, PI3K, and p-PI3K molecules in HT29 and DLD-1 cells. Furthermore, the combination of deoxyshikonin with LY294002, NVP-BEZ235, or MK-2206 improved its ability to suppress cell division, stop the cell cycle at G0/G1, and encourage apoptosis. Deoxyshikonin treatment significantly reduced the weight of the tumor tissue and the expression of mTOR, Akt, p-Akt308, PI3K, and p-PI3K in vivo in comparison to the control group [20]. Effectively, HO-3867 inhibited the development of OSCC cells. The sub-G1 phase is induced by HO-3867, according to the flow cytometry results. After analyzing the MAPK pathway, it was shown that the c-Jun N-terminal kinase (JNK)1/2 route is how HO-3867 causes cell apoptosis. Our findings indicate that HO-3867 is a potent anticancer drug because it causes human oral cancer cells to undergo apoptosis via the JNK1/2 pathway [21].

By increasing caspase-3/7 activity, Cyt C levels, and blocking PKM2 expression, glucose consumption, and lactate formation, deoxyshikonin decreased cell viability and accelerated apoptosis in AML cells. It inhibited the Akt/mTOR pathway, and when it was activated, DSK's effects on glycolysis, apoptosis, and viability were reversed. HO-1 and caspase cleavage were decreased when p38 kinase was inhibited, suggesting that tongue cancer cells undergo p38-dependent apoptosis. The anticancer potential of deoxyshikonin against KB oral squamous carcinoma cells is examined in this work. Its therapeutic promise in the treatment of OSCC requires more investigation and clinical studies[22]. Deoxyshikonin's ability to treat oral squamous cell cancer (OSCC) was investigated using a multidisciplinary approach that included database mining, network analysis, and route enrichment studies. About 38 genes were found to consistently overlap when these datasets were combined, suggesting that these genes may be important targets for deoxyshikonin in OSCC. Through network analysis, the regulatory linkages between deoxyshikonin and its probable targets were elucidated using the STITCH database. This demonstrated the importance of key genes in the development of oral cancer, including MCL1, SIRT1, IL6, IL10, CASP3, and CASP9. The top 6 targets were thoroughly investigated based on their network characteristics because of their significant roles in OSCC. This all-inclusive approach boosted confidence in the identification of deoxyshikonin-associated targets and

provided insightful information about the potential roles these targets may play in OSCC pathogenesis. The functional importance of these identified targets was further determined by pathway enrichment and gene ontology (GO) analysis. Highly enriched GO keywords associated with glycolytic processes, programmed cell death, gene expression control, and cell proliferation highlight their significance in OSCC biology, according to the analysis of g datasets.

KEGG pathway analysis also revealed significant enrichment in pathways like FoxO, PI3K-Akt, p53 signaling, and inflammatory mediator modulation. Numerous target genes were linked to cancer-related pathways, suggesting that deoxyshikonin might be involved in correcting these dysregulated pathways in oral cancer. In order to thoroughly assess the cytotoxic effects of deoxyshikonin on KB cells in the setting of oral cancer, our work used computational and experimental methodologies. Using the MTT and trypan blue tests, we looked at how various DSK doses affected the viability of KB cells over the course of 24 and 48 hours. The trypan blue test further supported these findings by demonstrating that deoxyshikonin treatment prevented KB cells from proliferating. Furthermore, we found that lactate generation and glucose absorption were sluggish in KB cells when we looked at how deoxyshikonin affected aerobic glycolysis. These results demonstrate DSK's ability to disrupt essential metabolic pathways connected to the growth of cancer and to function as a cytotoxic agent against oral cancer cells. By demonstrating lower levels of catalase (CAT) and SOD activity, the results revealed a role for deoxyshikonin in controlling ROS levels in KB cells. These findings shed light on deoxyshikonin's method of action and its use in the treatment of oral cancer. Moreover, DCF-DA staining revealed that KB cells treated with DSK had higher ROS activity. The significance of ROS modulation in cancer cell formation and the therapeutic potential of DSK in targeting ROS-mediated pathways in oral cancer are highlighted by this finding. The purpose of the study was to confirm that deoxyshikonin is a useful treatment for oral cancer by examining its impact on KB cells. Through the use of PI and DCFH-DA staining, cytomorphological analysis demonstrated that KB cells treated with DSK at IC50 doses for 24 and 48 hours exhibited significant morphological alterations, including blebbing, shrinkage, and cases of cell death.

Additionally, deoxyshikonin-treated KB cells had less ROS production than untreated cells, according to an examination of ROS activity, indicating that DSK can control ROS levels. Furthermore, deoxyshikonin treatment for 24 and 48 hours caused KB cells to undergo both early and late apoptosis; after 48 hours, nuclear staining with AO/EtBr demonstrated a marked increase in apoptotic processes. These findings provide valuable insight into the effects of deoxyshikonin on oxidative stress, cellular responses, and apoptotic processes in oral cancer cells, which may support the compound's potential therapeutic efficacy in treating the disease. After 24 and 48 hours, deoxyshikonin (DSK) treatment at IC50 doses in KB cells resulted in significant changes in the expression of apoptotic genes (CASP3 and CASP9) as well as in genes related to protein synthesis, cell division, and aerobic glycolysis such as IL6, IL10, MCL1, and SIRT1. These changes were detected by real-time PCR analysis. These findings imply that deoxyshikonin may control these genes and have an effect on the AKT1/mTOR signaling pathway, suggesting that it may be used to treat oral cancer. The connection between deoxyshikonin and proteins involved in apoptosis, metabolic regulation, and cell development is clarified by the molecular docking simulations used in our study. High binding energies in protein interactions set DSK apart, suggesting a robust affinity for targets such as IL6, IL10, MCL1, CASP3, CASP9, and SIRT1. By carefully analyzing binding interactions, DSK's hydrogen bonding with specific amino acid residues was found, providing more insight into the molecular mechanisms behind the drug's therapeutic effects. MD simulation analysis showed that RMSD and RMSF results confirmed the stability of protein ligand complexes. Deoxyshikonin exhibits promise as a novel therapy agent for oral cancer since it targets multiple pathways. To completely understand its impact on medicine delivery and efficacy, more study is necessary. Notwithstanding these disadvantages, deoxyshikonin seems to hold promise as a treatment option for oral cancer. Future studies should focus on enhancing the drug's distribution and formulation in order to increase its clinical usefulness.

Conclusion

Deoxyshikonin targets important molecular pathways, showing considerable therapeutic potential against oral squamous cell cancer (OSCC). It interacts firmly with important proteins and dramatically inhibits the proliferation of KB cancer cells, combining bioinformatics and experimental validation. By altering the AKT1/mTOR signaling pathway, deoxyshikonin increases oxidative stress, influences metabolic control, and encourages apoptotic cell death. With the help of experimental data and computational insights, these results show that deoxyshikonin is a promising treatment option for OSCC. Future directions for this research could entail combining treatments to increase effectiveness while minimizing adverse effects, which could result in the development of fresh strategies for the treatment of oral squamous cell carcinoma. Furthermore, tailored cancer treatment may be advanced by targeted therapy that makes use of deoxyshikonin's effects on the mTOR pathway. Further investigation is needed into the clinical translation of deoxyshikonin, its interactions with other signaling pathways, and its broader usefulness in cancer treatment in order to fully realize its therapeutic potential and improve patient outcomes.

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